

Receptor Density Is Key to the Alpha2/Beta Interferon Differential Activities[▽]

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Multiple type I interferons (IFN- α/β) elicit Jak/Stat activation, rapid gene induction, and pleiotropic effects, such as differentiation, antiviral protection, and blocks in proliferation, which are dependent on the IFN subtype and the cellular context. To date, ligand- and receptor-specific molecular determinants underlying IFN- α/β differential activities or potencies have been well characterized. To analyze cellular determinants that impact subtype-specific potency, human fibrosarcoma U5A-derived clones, exhibiting a gradient of IFN sensitivity by virtue of increasing receptor levels, were monitored for Jak/Stat signaling, gene induction, cell cycle lengthening, and apoptosis. In cells with scarce receptors, IFN- β was more potent than IFN- $\alpha 2$ in antiproliferative activity, while the two subtypes were equipotent in all other readouts. Conversely, in cells with abundant receptors, IFN- $\alpha 2$ matched or even surpassed IFN- β in all readouts tested. Our results suggest that the differential activities of the IFN subtypes are dictated not only by the intrinsic ligand/receptor binding kinetics but also by the density of cell surface receptor components.

A persistent question in the field of helically bundled cytokines concerns the molecular basis of intracellular signal activation following binding to cognate cell surface receptors. Typically, cytokine-induced dimerization of the receptor subunits is thought to trigger catalytic transactivation of the associated Jak tyrosine kinases. Phosphorylation of critical receptor tyrosine motifs by the activated Jak proteins allows recruitment and activation of downstream Stat effectors (25, 34). A clear distinction can be made between the short homodimeric Jak2-activating receptors, such as the growth hormone or the erythropoietin receptors, and the more complex heteromeric receptors. Among these latter is the type I interferon (IFN) receptor, a prototypic class 2 receptor, made of two subunits, each associated with a different Jak enzyme (29). IFNAR2 contains extracellularly two fibronectin III domains forming a well-defined cytokine binding module. The cytoplasmic region of IFNAR2 is 250 amino acids long, interacts with Jak1, and contains two principal Tyr-based Stat recruitment motifs (24, 35). IFNAR1 is made of a large ectodomain of four fibronectin III domains, not all involved in ligand binding, and a 100-amino-acid-long cytoplasmic region complexed with Tyk2 and subjected to ligand-induced ubiquitination driving receptor proteolysis (13, 14).

A large array of IFNs (over a dozen α subtypes and one β subtype) bind to this ubiquitously expressed receptor complex to induce rapid gene expression programs that elicit measurable antiviral responses and cell growth inhibition as well as cell context-specific functional changes (4, 31). Several studies have reported on differential activities of type I IFNs, but no unique function has ever been attributed to a given subtype (see references in reference 29). Thus, a differential can be

defined as a lack of correlation between two specific activities. For instance, depending on the cell system, IFN- $\alpha 2$ and IFN- β can exhibit equivalent antiproliferative potency or over a 100-fold difference in antiproliferative potency and nearly equipotency in antiviral activity. Since no overt differences are observed in the structure or stoichiometry of the ligand-receptor complex formed with different subtypes, the concordant view points to the way each IFN subtype engages the available receptors. Indeed, kinetic measurements of the interaction of IFN- $\alpha 2$ and IFN- β with receptor ectodomains have shown substantial differences. IFNAR2 represents the high-affinity subunit, toward which IFN- $\alpha 2$ exhibits nanomolar binding affinity and IFN- β exhibits ~ 100 pM binding affinity. Conversely, IFNAR1 is the low-affinity subunit, toward which IFN- $\alpha 2$ exhibits micromolar affinity and IFN- β ~ 50 nM affinity (19, 22). The contribution of the individual and combined affinities on ternary complex formation by either IFN subtype have been thoroughly studied (10, 26). However, how these dynamic parameters influence receptor function and translate into activation of Jak, recruitment of Stats and additional effectors, gene induction, and bioactivities remains ill defined.

Rather than focusing on ligand and receptor determinants, here we investigated the relationship between receptor subunit levels and IFN- $\alpha 2$ versus IFN- β signaling and functional outcomes (IFN- $\alpha 2/\beta$ differential potencies). Since we previously showed that no simple relationship between receptor levels and Jak/Stat signaling can be inferred by comparing different cell types (18), we have used a reductionist approach in a single cell type, from which we have engineered and studied clones expressing low or abundant receptor levels. We show that the density of receptors at the cell surface represents a critical determinant of the level of differential activity exhibited by two IFN subtypes.

MATERIALS AND METHODS

Cells, plasmids, and reagents. The IFN-unresponsive (IFNAR2-deficient) U5A mutant was derived from human fibrosarcoma 2FTGH cells and is described in

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TABLE 1. Antiproliferative response and receptor abundance in human cell lines

Cell line	Antiproliferative EC ₅₀ (pM)			Surface receptor level ^a		
	IFN-α2	IFN-β	IFN-α2/β	IFNAR1	IFNAR2	IFNAR2/R1
2fTGH	100,000	133	7,518	15	10	0.7
WISH ^b	3,000	50	60	22	18	0.8
MDA231 ^b	600	30	20	12	9	0.7
Daudi	0.15	0.15	1	80	200	2.5
U5-low/low	321	44	7.3	16	45	2.8
U5-low/hi	27	18	1.5	15	130	8.6
U5-hi/hi	1.4	6	0.24	45	100	2.2

^a Mean fluorescent intensity values from cytofluorimetric analyses.
^b EC₅₀s were taken from reference 12.

reference 17. All clones were cultured in Dulbecco's modified Eagle's medium and 10% heat-inactivated fetal calf serum. The PVN53 clone, described in reference 16, was used in this study and renamed "U5-low/low" for the sake of clarity. Transfections were performed with FuGENE6 (Roche Applied Science). IFNAR1 and IFNAR2 cDNAs were each subcloned into pMET7 vector (a gift of J. Tavernier, Ghent University). To obtain clone U5-low/hi, U5A cells were cotransfected with pMET-IFNAR2 and pSVpuro, selected in 0.3 µg/ml puromycin. Twenty-five puromycin-resistant clones were ring cloned and analyzed by fluorescence-activated cell sorter (FACS) for IFNAR2 expression compared to that of 2fTGH cells. To obtain clone U5-hi/hi, U5A cells were cotransfected with pMET-IFNAR1, pMET-IFNAR2, and pSVpuro. Eighty puromycin-resistant clones were analyzed by FACS for receptor expression compared to that of 2fTGH cells. Recombinant IFN-α2b was a gift of D. Gewert (Wellcome, United Kingdom), and IFN-β was from Biogen Idec (Boston, MA). IFNs were purified to specific activities of >10⁸ IU/mg of protein. IFN-α8-tail and IFN-HEQ were previously described (10, 26). MDA231 cells were obtained from P. Chavrier (Institut Curie, Paris). Pan-caspase inhibitor Z-VAD-FMK was purchased from R&D Systems.

FACS analysis. Surface receptor levels were monitored as described in reference 18, using monoclonal antibodies (MAbs) AA3 (BiogenIdec, Boston) and CD118 (PBL, Piscataway, NJ), which are specific for IFNAR1 and IFNAR2 respectively. Adherent cells were first detached in phosphate-buffered saline (PBS) with 5 mM EDTA and resuspended in PBS containing 3% fetal calf serum. Surface expression of Fas was measured by staining with allophycocyanin-

conjugated anti-CD95 MAb (clone DX2; BD Pharmingen). Samples were analyzed with a Becton Dickinson FACScan flow cytometer.

Competitive binding assay. The competitive binding assay was performed according to reference 12. Briefly, IFN-α2 was labeled with ¹²⁵I using the chloramine T iodination method, and the radiolabeled ligand was passed through Sephadex-G10 size exclusion column. Cells were seeded on 24-well plates (1.5 × 10⁵ cells/well) and 16 h later incubated for 30 min at 20°C with approximately 1 nM of ¹²⁵I-IFN-α2 (3 × 10⁵ cpm/well) alone or in the presence of unlabeled competitor IFN-α2 or IFN-β (doses ranging from 30 nM to 30 pM). Dilutions were made in threefold steps in Dulbecco's modified Eagle's medium and 2% fetal calf serum. Cells were washed twice in PBS to eliminate unbound IFN, trypsinized, and transferred into test tubes to measure bound ¹²⁵I-IFN-α2, using a γ counter (Packard). Each experiment, performed in triplicate, was repeated three times. Fifty percent inhibitory concentrations (IC₅₀s) were calculated using Prism software on normalized data from the three experiments (nine replicates).

Antiproliferative activity assay. Cells were seeded in 96-well plates at 5 × 10³ cells/well and incubated for 20 h. IFN was added, and incubation was continued for an additional 48 h. IFN doses ranged from 1 pM to 10 nM for U5-low/low cells and 0.1 pM to 1 nM for U5-low/hi and U5-hi/hi cells. Cells were washed with PBS, fixed with paraformaldehyde, and stained with 0.5% (wt/vol) crystal violet solution for 5 min. After removing the solution, plates were washed with deionized water and air dried. Crystal violet was solubilized with 1% (vol/vol) Triton X-100, and its absorbance was measured at 570 nm with a microplate reader. Each assay, performed in triplicate, was repeated three times. The specific activity of an IFN was expressed as the 50% effective concentration (EC₅₀) (Table 1). EC₅₀s were determined by fitting a sigmoidal curve to the dose-response curve using GraphPad Prism software.

Western blot. Cells were lysed in radioimmunoprecipitation assay buffer, and 30 µg of proteins was analyzed as described in reference 18. The following polyclonal antibodies (Abs) were used: anti-phospho-Tyr1054/Tyr1055 Tyk2 (Calbiochem); anti-phospho-Tyr689 Stat2; anti-phospho-Tyr701 Stat1, anti-phospho-Tyr705 Stat3, and anti-p48/IRF9 (a gift from D. Levy, New York University, NY); anti-pan Akt (Cell Signaling Technology, Beverly, MA); and anti-cleaved caspase 3 (Asp175) and anti-cleaved caspase 7 (Asp198) (Calbiochem). Anti-caspase-8 (1C12) was from Cell Signaling Technology. Signal was revealed with the ECL enhanced chemiluminescence Western blotting reagent (Pierce) or the more sensitive Western Lightning Chemiluminescence Reagent Plus (PerkinElmer).

Real-time quantitative PCR. Total RNA was purified with RNeasy columns (Qiagen). Reverse transcriptions were primed with random primers and per-

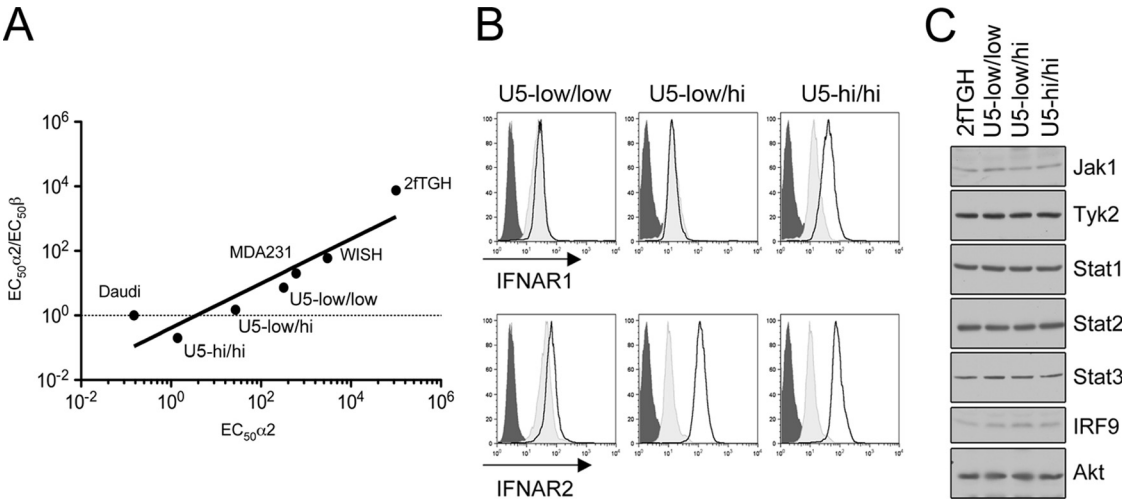


FIG. 1. (A) Graphic representation of the relative antiproliferative potencies of IFN-α2 and IFN-β in the human cell lines 2fTGH (fibrosarcoma), WISH (amniotic), MDA231 (breast cancer), and Daudi (B lymphoma) and the three U5-derived clones (U5-low/low, U5-low/hi, and U5-hi/hi) described in this study. EC₅₀s (pM) for IFN-α2 are plotted against the ratio of the EC₅₀ of IFN-α2 to that of IFN-β for each cell line (Table 1). (B) Levels of IFNAR1 and IFNAR2 in the three U5-derived clones (U5-low/low, U5-low/hi, and U5-hi/hi) and in the parental 2fTGH cells. Surface IFNAR1 and IFNAR2 were quantified by FACS analysis using MAbs AA3 and CD118, respectively. Dark gray area, isotypic control; light gray area, 2fTGH; black line, U5-low/low, U5-low/hi, or U5-hi/hi. Of note, these clones exhibited similar forward scatter values, as determined by FACS analysis, indicating that the increases observed are due to changes in surface receptor density and not cell volume. (C) Analysis of the expression level of Jak/Stat pathway components in 2fTGH cells and the U5-derived clones. Total cell lysates (30 µg) were analyzed by Western blotting with the indicated Abs. Loading was evaluated by measuring Akt levels.

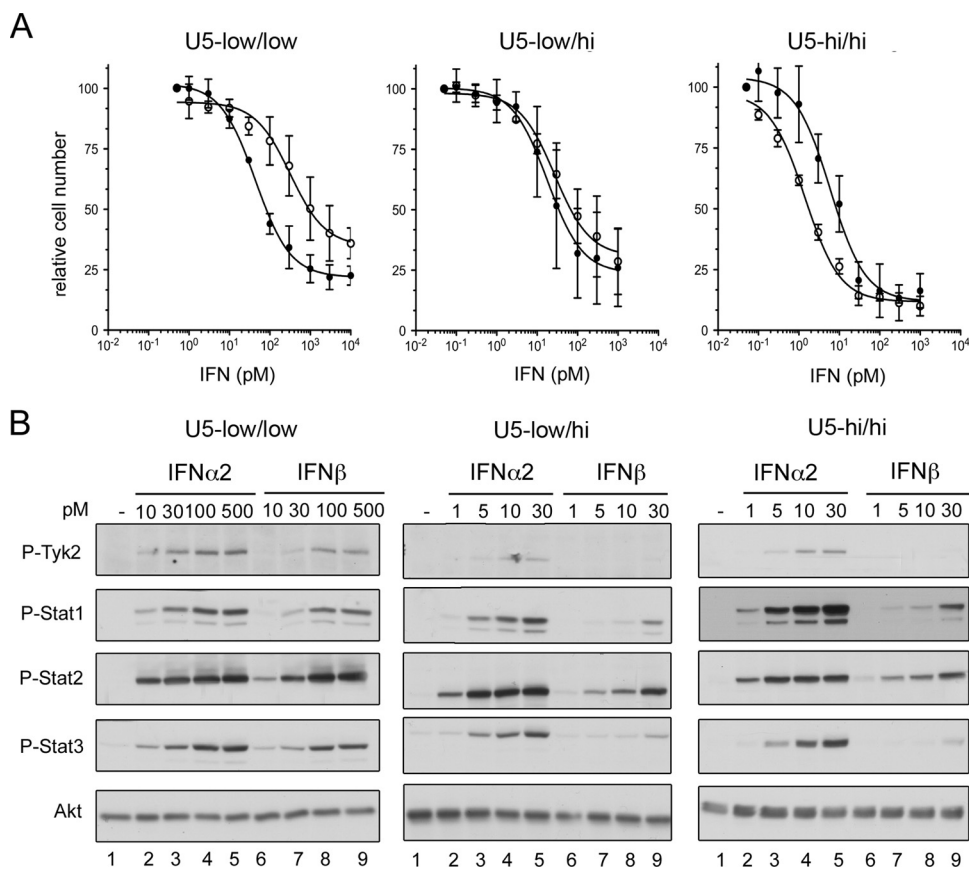


FIG. 2. Antiproliferative and Jak/Stat activation potencies of IFN- α 2 and IFN- β in U5-low/low, U5-low/hi, and U5-hi/hi cells. (A) Antiproliferative dose-response curves of U5-low/low, U5-low/hi, and U5-hi/hi cells treated for 48 h with IFN- α 2 or IFN- β . Cell density was measured with crystal violet staining. Note the higher doses of IFN used for U5-low/low cells. Open circles, IFN- α 2; filled circles, IFN- β . Error bars represent the standard deviation of the mean of three independent experiments, each performed in triplicate. (B) Dose-dependent profiles of tyrosine phosphorylation of Tyk2 and Stat1/2/3, measured in cells treated for 15 min with the indicated doses of IFN- α 2 or IFN- β . Note that for U5-low/low cells, IFN doses ranged from 10 to 500 pM. For the other two clones, doses were from 1 pM to 30 pM. Lysates (30 μ g) were analyzed by Western blotting. Loading was evaluated by measuring Akt levels. To evaluate differences, for U5-low/low cells, compare lanes 2 and 7; i.e., 10 pM IFN- α 2 versus 30 pM IFN- β . For U5-low/hi cells, compare lanes 3 and 9; i.e., 1 pM IFN- α 2 versus 30 pM IFN- β . The results shown are representative of three independent experiments.

formed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using the TaqMan gene expression assay technology (Applied Biosystems) for *6-16* (catalog no. Hs00242571) and *MxA* (catalog no. Hs00182073). Each sample was run in triplicate, normalized to the 18S RNA amplification level in the same sample, and calculated relative to expression of the target gene in unstimulated cells.

Cell cycle analysis. Cells were seeded at 3×10^5 cells in 60-mm plates and the next day stimulated with different doses of IFN- α 2 or IFN- β . Twenty-four and 48 h later, cells were fixed in 70% ice-cold ethanol. DNA staining was performed by incubating cells at room temperature in PBS containing 0.18 mg/ml propidium iodide and 0.4 mg/ml DNase-free RNase (Sigma Aldrich, St. Louis, MO). In all experiments, cells were acquired on a Becton Dickinson FACSscan flow cytometer and analyzed using the Flowjo software.

RESULTS

Antiproliferative potency of IFN- α 2 and IFN- β in human cell lines. To address the question of the molecular events behind the differential activities of type I IFN subtypes—of which the antiproliferative activity is a convenient readout—we first compared the antiproliferative potencies of IFN- α 2 and IFN- β on three cell lines of epithelial origin (2fTGH, WISH,

and MDA231) and on B-lymphoma Daudi cells. As summarized in Table 1, the two subtypes were equipotent only in Daudi cells, and overall, antiproliferative potencies appeared to increase with the abundance of surface receptors measured by cytofluorimetry. By plotting, for each cell line, the potency (expressed as EC₅₀) of IFN- α 2 against the ratio of the EC₅₀ of IFN- α 2 to that of IFN- β , we observed that the IFN- α 2/ β differential diminished in highly sensitive cells, suggesting an inverse correlation between IFN- α 2 sensitivity and differential potency (Fig. 1A).

To get more insights into the relation between receptor abundance and the IFN- α 2/ β differential, we focused on a single cell type from which we derived clones exhibiting a gradient of IFN sensitivity. For this, IFNAR2-deficient U5A cells were stably transfected with IFNAR2 and/or IFNAR1 and clones were analyzed for their surface receptor level relative to parental 2fTGH cells. Three representative clones were chosen, and for convenience, these were named “U5-low/low,” “U5-low/hi,” and “U5-hi/hi,” in relation to their level

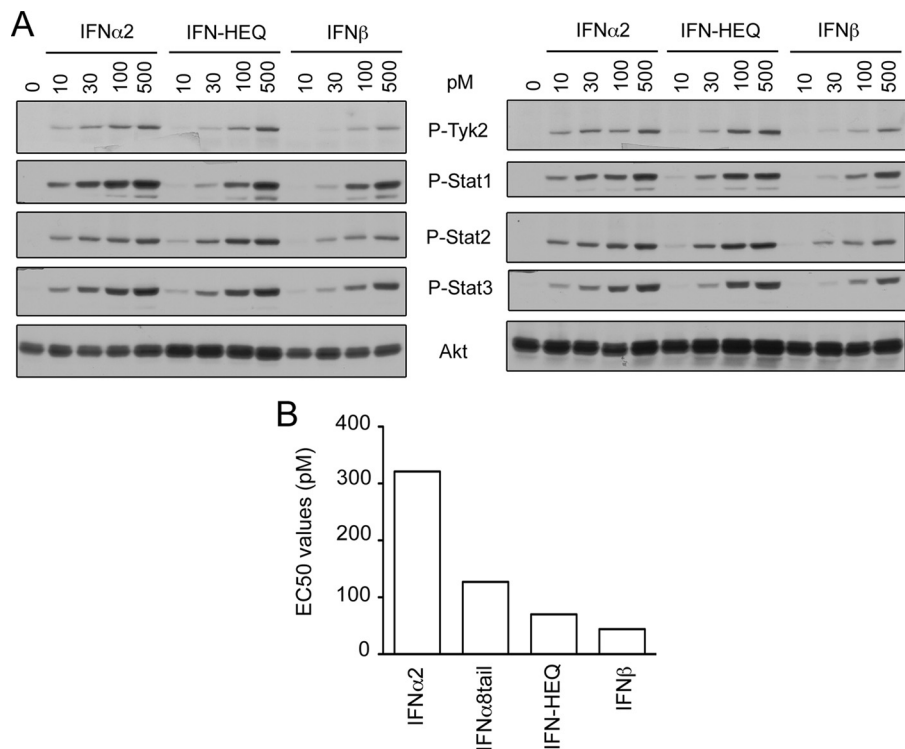


FIG. 3. Jak/Stat activation and antiproliferative potencies of two IFN- α 2 mutants on U5-low/low cells. (A) Dose-response profiles of phosphorylation of Tyk2 and Stat1/2/3 in U5-low/low cells treated for 15 min with IFN- α 2, IFN- α 8-tail or IFN- β (left), and IFN- α 2, IFN-HEQ, or IFN β (right). Lysates (30 μ g) were analyzed by Western blotting. Loading was evaluated by measuring Akt levels. Results are representative of at least three independent experiments. (B) Antiproliferative potencies of IFN- α 2, IFN-HEQ, IFN- α 8-tail, and IFN- β measured on U5-low/low cells treated for 48 h. EC₅₀s (pM) from a representative experiment are shown.

(low, medium, or high) of IFNAR1 or IFNAR2 (Fig. 1B). The three clones expressed comparable levels of Jak/Stat pathway components (Fig. 1C).

First, we compared the antiproliferative potencies of IFN- α 2 and IFN- β in each clone. Cells were stimulated with different IFN doses, and cell density was measured after 48 h (Fig. 2A). As summarized from the EC₅₀s shown in Table 1, IFN- β was found to be sevenfold more potent than IFN- α 2 in the U5-low/low clone. In the clones with higher receptor levels, the overall IFN potency was increased and, importantly, the antiproliferative differential was reduced. In fact, no IFN- α 2/ β differential could be measured in U5-low/hi cells, while a clear differential was observed in U5-hi/hi cells, with, however, IFN- α 2 exhibiting fourfold-higher potency than IFN- β (Fig. 2A and Table 1). These results demonstrate that an increase in the overall cell sensitivity to IFN, achieved through higher receptor expression, leads to a reduction or inversion in the IFN- α 2/ β antiproliferative differential.

In cells with abundant receptors, IFN- α 2 is more potent than IFN- β in Jak/Stat activation. Given the different antiproliferative potencies of the two IFN subtypes measured in the three clones, we analyzed at several steps the intracellular events triggered by each IFN. For immediate signaling, we monitored tyrosine phosphorylation of Jak/Stat proteins after 15 min of stimulation (Fig. 2B). Importantly, the range of IFN doses used was adjusted for each clone so as to obtain signals of similar intensity among the clones, at least for one dose. By comparing signal intensities of phospho-Tyk2, -Stat1, and

-Stat3, IFN- α 2 appeared to be 2- to 3-fold more potent than IFN- β in the U5-low/low cells, around 6-fold more potent than IFN- β in U5-low/hi cells, and 30-fold more potent in U5-hi/hi cells (Fig. 2B). Differences in phosphorylated Stat2 levels were less evident. These results showed that, in cells expressing abundant receptors, IFN- α 2 was markedly more potent than IFN- β in Jak/Stat activation and concurrently the IFN- α 2/ β antiproliferative differential was abolished or even reversed. On the other hand, in U5-low/low cells signaling by the two subtypes was almost comparable, even though in these cells IFN- β exerted a stronger antiproliferative effect.

Higher potency of IFN- α 2 is related to its weaker binding. From comparing the antiproliferative EC₅₀s measured in the three clones and summarized in Table 1, it emerged that, in cells with abundant receptors, the gain of potency was markedly higher for IFN- α 2 than for IFN- β . We therefore analyzed how this relates to the receptor binding and assembly properties of the ligand. For this, we made use of two IFN- α 2 mutants that were engineered to exhibit higher receptor binding affinity than the natural IFN- α 2. One mutant, IFN- α 8-tail, was described to possess higher binding affinity to the IFNAR2 chain than IFN- α 2 (26). The other mutant, IFN-HEQ, was described to have higher binding affinity to IFNAR1 than IFN- α 2 (10). In U5-low/low cells, the two mutants were slightly less potent than IFN- α 2 in signaling (Fig. 3A), and as shown in WISH cells (10, 26), they were more potent than IFN- α 2 in antiproliferative activity (Fig. 3B). On the other hand, in U5-hi/hi cells the two mutants were found to be less potent than IFN- α 2 in both

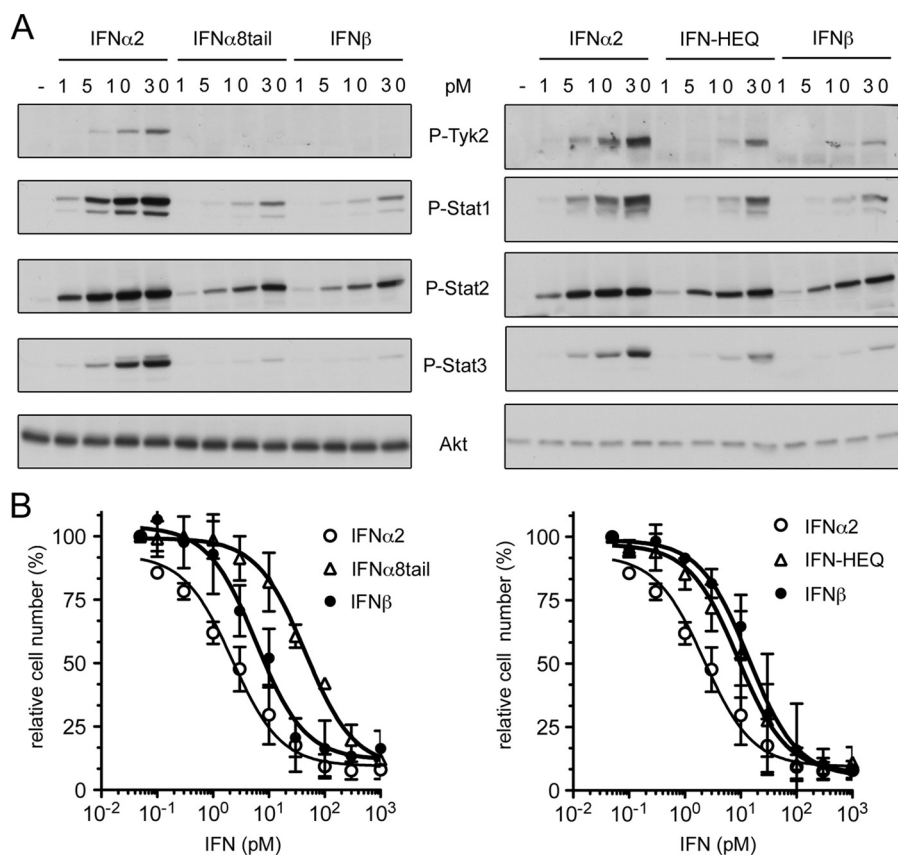


FIG. 4. Jak/Stat activation and antiproliferative potencies of two IFN- α 2 mutants on U5-hi/hi cells. (A) Dose-response profiles of phosphorylation of Tyk2 and Stat1/2/3 in U5-hi/hi cells treated for 15 min with IFN- α 2, IFN- α 8-tail, or IFN- β (left panels) and with IFN- α 2, IFN-HEQ, or IFN- β (right panels). Lysates were analyzed as described in the legend to Fig. 3. Note that IFN- α 8-tail behaved like IFN- β , and IFN-HEQ was two- to threefold more potent than IFN- β . (B) Antiproliferative dose-dependent curves of U5-hi/hi cells treated for 48 h with IFN- α 2, IFN- α 8-tail and IFN- β (left) or treated with IFN- α 2, IFN-HEQ, and IFN- β (right). Error bars represent the standard deviation of the mean of three independent experiments, each performed in triplicate. The EC_{50} s are 1.4 pM for IFN- α 2, 6 pM for IFN- β , 45 pM for IFN- α 8-tail, and 8 pM for IFN-HEQ.

signaling and antiproliferative activities (Fig. 4). Overall, these results suggested that the higher potency of natural IFN- α 2 in cells with abundant receptors is related to its weaker binding.

To corroborate this, we performed binding studies using 125 I-IFN- α 2. We compared the apparent binding affinity of the two IFN subtypes by competitive binding assays (12). U5-low/low and U5-hi/hi cells were incubated with 125 I-IFN- α 2 alone or mixed with unlabeled IFN- α 2 or IFN- β competitor at 30 nM to 0.03 nM doses. Specific cell-bound 125 I-IFN- α 2 was quantified, and IC_{50} s were obtained (Fig. 5A). The capacity of IFN- α 2 to displace itself was slightly higher (2 \times) in U5-hi/hi cells compared to U5-low/low cells, while the capacity of IFN- β to displace IFN- α 2 remained constant in the two clones. The important point is that in U5-hi/hi cells, IFN- β was still remarkably more potent than IFN- α 2 in displacement activity in spite of being less potent in antiproliferative activity. Moreover, IFN- β retained the ability to downregulate IFNAR2 (Fig. 5B) (11, 18). These results strongly suggest that the more robust signaling and antiproliferative activity exhibited by IFN- α 2 in cells with abundant receptors is related to its weaker binding property and may involve sequential receptor engagement (see Discussion).

ISGF3-dependent gene induction in U5-derived clones. As a second readout of IFN action in the three clones, we measured the level of transcripts of two IFN-stimulated genes (ISGs), *Mx4* and *6-16*, whose induction is dependent on the transcription complex ISGF3 (Stat1/Stat2/IRF9). Cells were stimulated for 8 h with two IFN doses appropriately chosen for each clone: one dose corresponding to the lowest antiproliferative EC_{50} (Table 1) and one higher dose (Fig. 6). In U5-low/low cells, IFN- α 2 and IFN- β induced the *Mx4* and the *6-16* genes to a similar extent at the two doses tested. A similar behavior was observed in U5-low/hi cells. On the other hand, in U5-hi/hi cells at both doses IFN- α 2 induced higher transcripts than IFN- β . Transient reporter assays, using an IFN-stimulated response element-driven luciferase gene, were also performed in the three clones and confirmed these results (data not shown). In conclusion, our analyses showed that in U5-low/low cells, with scarce receptors, at the antiproliferative EC_{50} of IFN- β dose (50 pM), the two IFN subtypes were virtually equipotent in ISG induction. Thus, the higher antiproliferative potency of IFN- β in these cells did not correlate with either higher Jak/Stat signaling or higher ISGF3-dependent gene induction. Interestingly, in U5-hi/hi cells, expressing more receptors, differ-

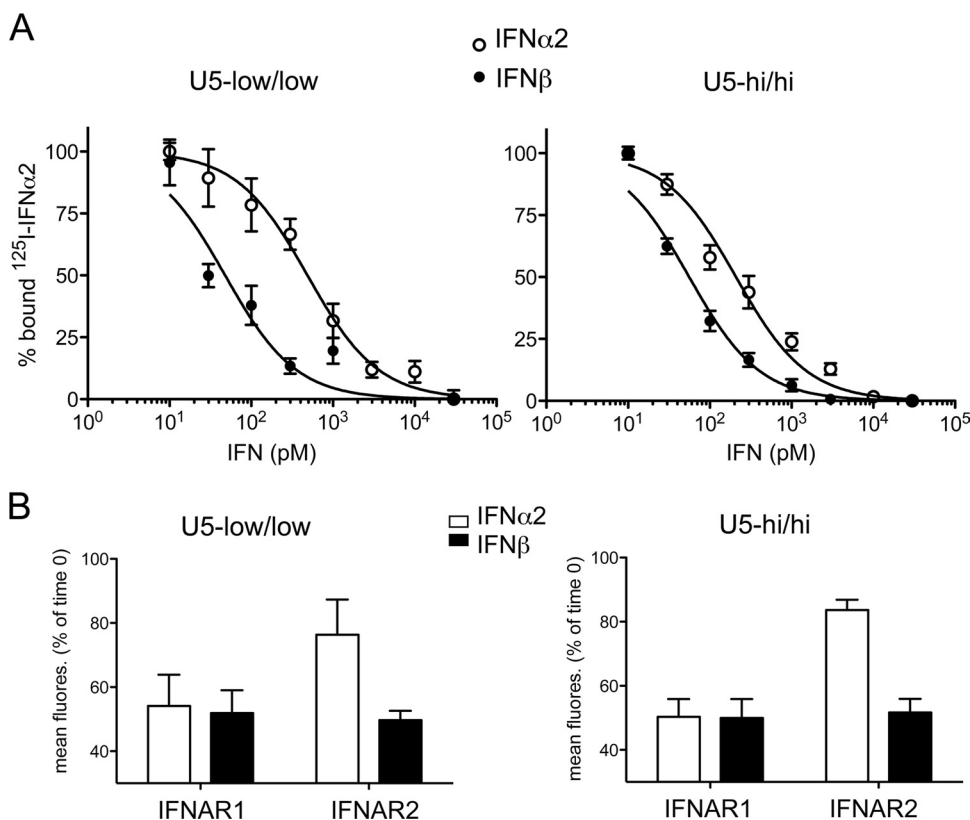


FIG. 5. Displacement of ^{125}I -IFN- α 2 and downregulation of IFNARs by IFN- α 2 and IFN- β in U5-low/low and U5-hi/hi cells. (A) Displacement of ^{125}I -labeled IFN- α 2 by unlabeled IFN- α 2 (open circle) or IFN- β (closed circle) was measured on U5-low/low cells (left) and U5-hi/hi (right) cells, as described in Materials and Methods. Specific cell-bound ^{125}I -IFN- α 2 was quantified and plotted. Error bars represent the standard errors of the mean of three independent experiments, each performed in triplicate. The IC_{50} s for U5-low/low cells are 487 pM for IFN- α 2 and 48 pM for IFN- β . The IC_{50} s for U5-hi/hi cells are 209 pM for IFN- α 2 and 55 pM for IFN- β . (B) IFNAR1 and IFNAR2 decay from the cell surface was measured by flow cytometry in U5-low/low (left) and U5-hi/hi (right) cells treated with IFN- α 2 or IFN- β (500 pM) for 60 min in the presence of cycloheximide. Percentages of the mean fluorescence (fluores.) at time zero (mean \pm standard error for at least three experiments) are shown.

ential gene induction was clearly observed in favor of IFN- α 2 at the 1 pM EC_{50} dose correlating with more robust Jak/Stat signaling (Fig. 2B).

IFN-induced cell cycle lengthening and apoptosis in U5-derived clones. In several tumor cell lines, type I IFNs have been shown to induce lengthening of the cell cycle S phase (7, 32). Thus, we examined the effect of the two IFN subtypes on cell cycle. The three clones were incubated for 24 h with IFN doses which, as for gene induction analyses, covered the IFN- α 2 and IFN- β antiproliferative EC_{50} range for each clone. The percentage of cells present in the different phases of the cell cycle was measured by FACS. In the absence of stimulation, all clones exhibited a similar distribution profile, with $\sim 47\%$ of cells in S phase. This percentage increased to about 70% when cells were incubated with saturating IFN doses (Fig. 7A). No IFN- α 2/ β differential was observed in U5-low/low cells at any of the doses tested. In U5-low/hi cells incubated with the suboptimal 1 pM dose, IFN- α 2 induced a slightly higher S phase accumulation. On the other hand, in U5-hi/hi cells IFN- α 2 was markedly more potent than IFN- β .

It has been shown that after prolonged treatment, IFN can induce apoptosis (5, 21). Since no IFN- α 2/ β differential effect on cell cycle was observed in U5-low/low cells at 24 h, we asked whether a differential apoptotic effect could be observed at a

later time of stimulation. Thus, cells were incubated for 48 h with the same doses as above and the percentage of apoptotic cells (cells in sub- G_1 phase) was measured by FACS (Fig. 7B). Interestingly, in U5-low/low cells IFN- β induced a twofold-higher level of apoptosis than IFN- α 2. In U5-low/hi cells, no consistent differential was observed, while in U5-hi/hi cells IFN- α 2 induced more apoptosis than IFN- β . These results were confirmed using the annexin V-propidium iodide assay (data not shown). In conclusion, in cells with abundant receptors, where low doses of IFN- α 2 induced more robust Jak/Stat phosphorylation and gene induction than IFN- β , IFN- α 2 attained and even surpassed IFN- β in apoptotic and antiproliferative potencies. On the other hand, in U5-low/low cells with scarce receptors, IFN- β exhibited higher apoptotic potency that is likely to contribute to, or be the basis of, its stronger antiproliferative potency (Fig. 1).

IFN- β induces higher caspase processing and surface Fas expression. Next, we focused on U5-low/low cells and addressed the basis by which IFN- β induced a higher level of apoptosis than IFN- α 2 in these cells. Two major pathways have been described for induction of apoptosis: one initiating at the level of cell surface death receptors and the other involving mitochondrial activation and cytochrome *c* release. Since both pathways are known to trigger caspase cascades, we analyzed

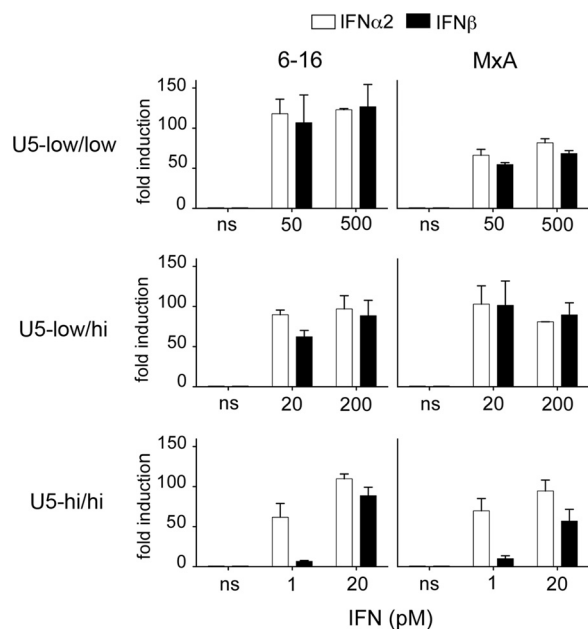


FIG. 6. Induction of the *MxA* and *6-16* genes by IFN- α 2 and IFN- β in U5-low/low, U5-low/hi, and U5-hi/hi cells. Induction of the *MxA* gene is shown in the left panels, and induction of the *6-16* gene is shown in the right panels in U5-low/low cells (upper), U5-low/hi cells (middle), and U5-hi/hi cells (bottom). Cells were left nonstimulated (ns) or were stimulated for 8 h with IFN- α 2 (white) or IFN- β (black). Note that, for each clone, one IFN dose corresponds to the lower antiproliferative dose (EC_{50}) and the other is 10-fold higher. Thus, U5-low/low cells (antiproliferative EC_{50} of IFN- β , 44 pM) were treated with 50 pM and 500 pM, U5-low/hi cells (antiproliferative EC_{50} of IFN- β , 18 pM) were treated with 20 and 200 pM, and U5-hi/hi cells (antiproliferative EC_{50} of IFN- α 2, 1.4 pM) were treated with 1 and 20 pM doses. Transcripts were measured by quantitative reverse transcription-PCR; values were normalized to simultaneously quantified 18S transcripts. For each clone, the ratios between treated and nonstimulated samples are shown, taking as 1 the ratio in nonstimulated samples. Each value represents the mean of three independent experiments, each performed in triplicate.

the extent of caspase processing induced by IFN- α 2 and IFN- β . In kinetic experiments, caspase activation was found to peak at around 36 h of stimulation (data not shown). Thus, U5-low/low cells were treated with 10 pM to 500 pM of IFN- α 2 or IFN- β for 36 h and the processing of caspases 3, 7, and 8 was analyzed by Western blotting. As shown in Fig. 8A, a higher level of cleavage products of the three caspases was detected in response to IFN- β . Cells were also coincubated with IFN and the pan-caspase inhibitor Z-VAD-FMK. As shown in Fig. 8B, the level of apoptosis induced by either IFN subtype was similarly affected, confirming the involvement of caspases in both contexts. Activation of caspase 8 is initiated by its recruitment to death receptors, notably Fas, via the Fas-associated death domain protein, or FADD. Since we and others have previously shown that type I IFNs sensitize cells to Fas-induced apoptosis (6, 28), we monitored surface levels of Fas after different times of stimulation with either IFN- α 2 or IFN- β . As shown in Fig. 8C, within the first 8 h the level of Fas was barely affected, whereas at later times Fas was more strongly upregulated in response to IFN- β . Altogether, these data provide evidence that in cells with scarce receptors, such as U5-low/low

or WISH cells (data not shown), the more potent growth inhibitory effect of IFN- β is exerted at the level of apoptosis, via, at least in part, upregulation of Fas and caspase 8 processing.

DISCUSSION

It is well documented that affinity differences in receptor recognition are at the origin of the specific bioactivities of different IFN subtypes (reviewed in reference 29). It is unknown, however, how these differences impact the intensity and diversification of signaling cascades, gene expression programs, and biological outputs. To address the mechanistic basis of the IFN- α 2/ β differential responses, we have measured a number of key intracellular events induced by two IFN subtypes in cells of common origin which differ only in receptor abundance. To measure receptor activation, we quantitated Jak/Stat phosphorylation as the earliest detectable signaling event as well as gene induction, cell cycle lengthening, and apoptosis in a dose-dependent fashion. The main observations of this comparative study are hereafter summarized. In U5-low/low cells, with a twofold-higher level of IFNAR2 than parental 2fTGH cells, the two IFN subtypes are virtually equipotent in Jak/Stat phosphorylation, gene induction, and cell cycle lengthening. However, as seen in WISH cells, IFN- β exhibits a higher antiproliferative potency than IFN- α 2, demonstrating that IFN- β is able to bring about a unique change toward the antigrowth effect. In cells with abundant receptors (U5-low/hi and U5-hi/hi), IFN- α 2 equals or even becomes more potent than IFN- β in all readouts. These data confirm the inverse correlation between the IFN- α 2/ β differential and overall IFN sensitivity suggested from comparing cells of different lineages (Fig. 1A).

How can this be explained mechanistically? Although antiproliferative potency cannot be simply correlated to Stat activation potency, the quantitative analysis of early signaling is informative. Indeed, in cells with scarce receptors, the two subtypes are equally potent in Jak/Stat activation, while in cells with abundant receptors IFN- α 2 is more potent (Fig. 2). In vitro measurements of the ligand-receptor interaction have shown large differences in the binding affinity of the two ligands to the receptor chains (see the introduction). To analyze how the binding affinity of the ligand contributes to the differentials observed, we have used two IFN- α 2 mutants that exhibit increased affinity to one or the other receptor chain (10, 26). Thus, we could show that in cells with abundant receptors both IFN- α 2 mutants were less potent in Jak/Stat phosphorylation and growth inhibition than natural IFN- α 2 (Fig. 4), suggesting that the low-affinity ligand is more fit to early signal activation.

In conclusion, even though IFN- α 2 presents an apparent binding affinity lower than that of IFN- β (Fig. 5A), it can possess similar or higher specific bioactivities in cells with abundant receptors. This apparent paradox can be explained if one takes into account also the stability of the ternary complexes formed by IFN- α 2 and IFN- β . Indeed, from measuring the rate constants of ternary complex assembly on artificial membranes, the half-life of the ternary complex was found to be considerably higher for IFN- β (100 s) than for IFN- α 2 (1 to 5 s) (8, 27). Thus, IFN- α 2 is likely to engage in short-lived ternary complexes and IFN- β in long-lived ternary complexes.

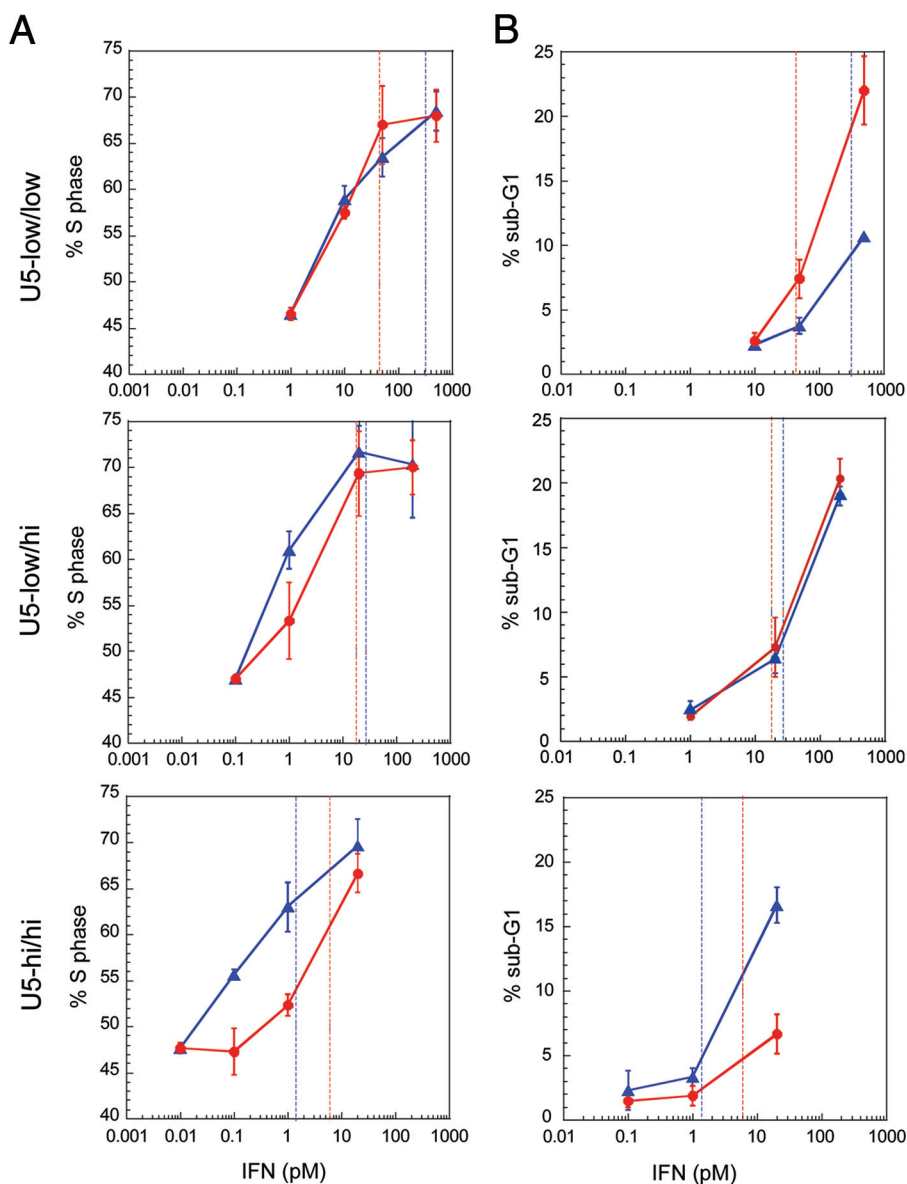


FIG. 7. Cell cycle lengthening and apoptotic potencies of IFN- α 2 and IFN- β in U5-low/low, U5-low/hi, and U5-hi/hi cells. (A) The percentage of cells (U5-low/low, U5-low/hi, and U5-hi/hi) that accumulated in S phase after 24 h of stimulation with the indicated doses of IFN- α 2 (triangles) or IFN- β (circles) was measured by flow cytometry. Each value represents the mean of three independent experiments. Vertical dotted lines correspond to the antiproliferative EC_{50} s. (B) Percentage of cells (U5-low/low, U5-low/hi, and U5-hi/hi) present in the sub-G₁ phase after 48 h of stimulation with the indicated doses of IFN- α 2 (triangles) or IFN- β (circles). Values were obtained from flow cytometry analysis. Each value represents the mean of three independent experiments. Vertical dotted lines correspond to the antiproliferative EC_{50} s.

These properties are somehow reminiscent of the behavior of the T-cell receptor (TCR) which exhibits high sensitivity and specificity to peptide-major histocompatibility complex ligands (1). One model proposed to explain the high TCR sensitivity is serial triggering, whereby a ligand of low affinity can sequentially engage multiple TCRs and thus amplify the signal (9, 30). An activation mode analogous to the one described for the TCR system could be invoked for the type I IFN receptor system. In cells with scarce receptors, engagement of most, if not all, receptors is likely to occur and the half-life of the ternary complex will discriminate between the two IFN subtypes and determine the signaling outcomes. Thus, the long-

lived complexes formed with IFN- β will be able to induce a signal or signals which ultimately reinforce apoptosis. In cells with abundant receptors, the number of ternary complexes formed, rather than their half-life, will be critical. In this context, IFN- α 2 will be advantaged by sequentially triggering multiple receptors, by virtue of its lower binding activity/affinity. This will amplify Jak/Stat signaling and downstream gene induction (see also the model in Fig. 9).

Another system in which two ligands share the same receptor and serial engagement could be invoked is the interleukin-4 (IL-4)/IL-13 system, which signals through the Jak/Stat cascade. On nonhematopoietic cells, both IL-4 and IL-13 bind to

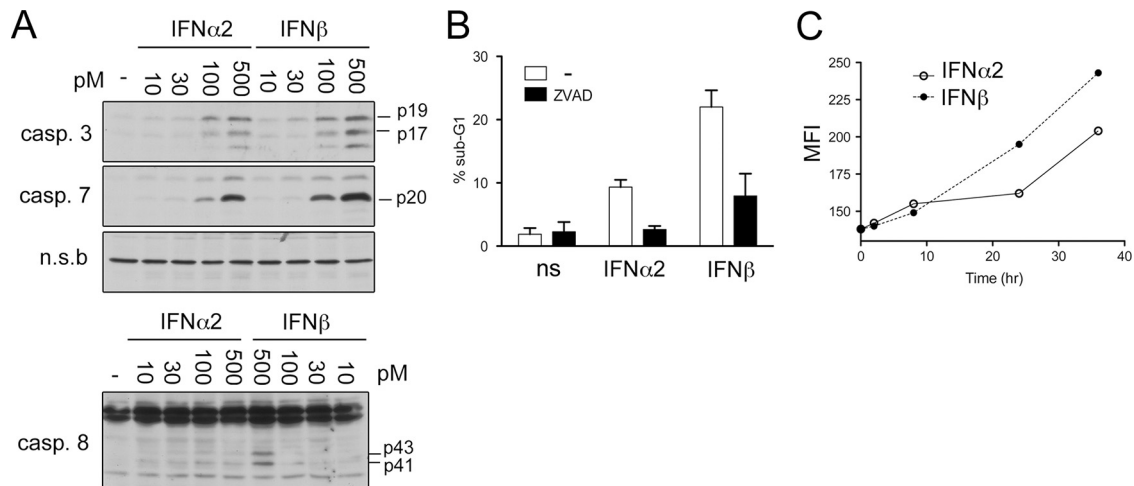


FIG. 8. Activation of caspases and induction of Fas by IFN- α 2 and IFN- β in U5-low/low cells. (A) U5-low/low cells were left untreated or treated with the indicated dose of IFN- α 2 or IFN- β for 36 h. Lysates (30 μ g) were analyzed by Western blotting for the presence of cleavage products of caspase (casp.) 3 (p19/p17), caspase 7 (p20), and caspase 8 (p43/p41). A nonspecific band (n.s.b) was used as the loading control. Note in the bottom panel the different order of sample loading. (B) U5-low/low cells were left untreated or were treated for 36 h with 500 pM of IFN- α 2 or IFN- β in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK (50 μ M). The percentage of cells in sub-G₁ was measured by flow cytometry. Each column represents the mean value of three independent experiments. ns, nonstimulated. (C) The surface level of Fas was monitored by flow cytometry on U5-low/low cells, untreated or treated for 4, 8, 24, and 36 h with 500 pM of IFN- α 2 or IFN- β . The median fluorescence intensities (MFI) obtained in one of three independent experiments are shown.

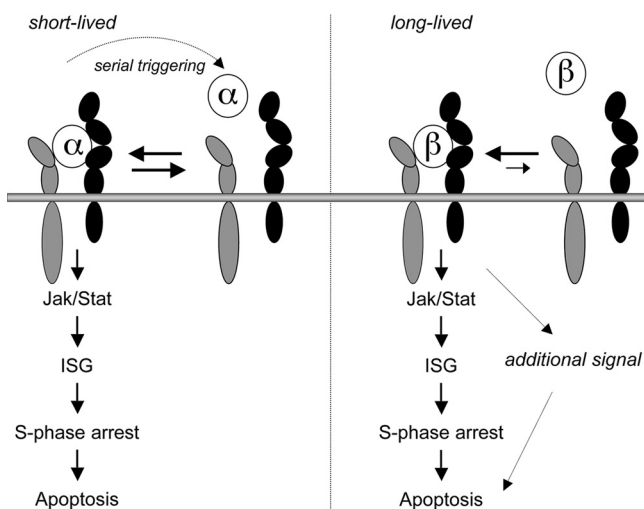


FIG. 9. Model of receptor activation by IFN- α 2 versus IFN- β and signaling outcomes. The model is based on reported in vitro parameters of binary and ternary complex assembly (see references in text) and on the present cell-based analyses. The left panel shows that a short-lived ternary complex composed of IFN- α 2, IFNAR2 (gray), and IFNAR1 (black) is fully fit to activate the Jak/Stat pathway, induce ISG expression, block cells in S phase, and induce a low level of apoptosis. Under the condition of a nonlimiting receptor, the rapidly dissociating ternary complexes release IFN- α 2, which serially engages unoccupied receptors. This will amplify Jak/Stat activation and downstream events, leading to increased apoptosis. In other terms, in this model, the effect of IFN- α 2 is not proportional to the number of occupied receptors but to the number of IFN-receptor associations per unit of time. In opposition, as illustrated in the right panel, the slowly dissociating IFN- β forms a more stable ternary complex than IFN- α 2, which translates in a single quantum of Jak/Stat activation. However, it is predicted that the stable IFN- β -receptor complexes activate additional proapoptotic signals which determine the more potent antigrowth effect of IFN- β measured in cells with low-density receptors. The model could accommodate other IFN subtypes, whose competence to form a tight ternary complex may vary.

a complex made of IL-4 receptor α (IL-4R α) and IL-13R α 1. The binding affinity of each cytokine for the two chains differs. IL-13 uses as a driver the IL-13R α 1 chain (high affinity) and as a trigger IL-4R α (low affinity), whereas IL-4 uses IL-4R α as the driver and IL-13R α 1 as the trigger. Although the complex with IL-13 appears to be the most stable by virtue of a higher affinity of the IL-13/IL-13R α 1 binary complex for IL-4R α , IL-4 was reported to be more potent than IL-13 at stimulating phosphorylation of Stat6 (15, 33). This unexpected result may underlie serial triggering, whereby the number of ternary complexes induced by IL-4 is higher than that induced by IL-13 and, as a consequence, IL-4 would activate Stat6 more efficiently.

Here we have also shown that the differential antiproliferative potency of IFN- α 2 and IFN- β measured in U5-low/low or in WISH cells is exerted at the level of apoptotic signals and is mediated, at least in part, by differential upregulation of Fas. We can only speculate on the mechanism by which IFN- β promotes higher surface Fas, as this question goes beyond our present study. It has been described that Fas localizes in intracellular stores (3) and that, upon proapoptotic stimuli, such as IFN- γ , it traffics to the plasma membrane (2). Therefore, an attractive possibility is that IFN- β somehow affects the intracellular traffic of proapoptotic receptors, such as Fas. In support of this, we found no evidence of Fas mRNA induction by either IFN subtype (data not shown), suggesting that IFN- β may act posttranscriptionally. Additionally, caspase 9, which is a measure of mitochondrion-dependent apoptosis, was activated to the same extent by the two IFN subtypes (data not shown), suggesting that IFN- β specifically sensitizes cells toward the extrinsic pathway. Overall our data point to a model whereby IFN- β , by assembling tighter complexes, may act upon additional signals, such as phosphatidylinositol 3-kinase/Akt, that somehow affect surface expression of proapoptotic receptors (20, 23).

Piehlér's group has recently shown that the two IFN subtypes form ternary complexes with similar architectures. Moreover, using fluorescence spectroscopy Piehlér's group also showed that the ectodomain of IFNAR1 undergoes a substantial rearrangement of the membrane-distal domains upon IFN binding (27). Future work will therefore need to address the possibility that in live cells this conformational change represents a signature toward diversification of signals and biological outputs.

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